PERFLUOROOCTANESULFONATE, POTASSIUM SALT (PFOS): A 96-HOUR STATIC-RENEWAL ACUTE TOXICITY TEST WITH THE SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-146A

ENVIRONMENTAL LABORATORY PROJECT NUMBER: U2723

U. S Environmental Protection Agency
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AUTHORS:

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STUDY INITIATION DATE: May 16, 2001

STUDY COMPLETION DATE: January 7, 2002

Submitted to

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Environmental Laboratory
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: Perfluorooctanesulfonate, Potassium Salt (PFOS): A 96-Hour Static-Renewal Acute Toxicity Test with the Sheepshead Minnow (*Cyprinodon variegatus*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-146A

STUDY COMPLETION: January 7, 2002

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

STUDY DIRECTOR:

Susan J. Palmer
Senior Biologist

07 January 2002
Date

SPONSOR APPROVAL:

Sponsor

1/10/02
Date

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Initial Trial 454A-146:			
Test Substance Preparation	July 6, 2001	July 6, 2001	July 11, 2001
Matrix Fortifications	July 10, 2001	July 11, 2001	July 13, 2001
Observations and Analytical Sampling	July 12, 2001	July 12, 2001	July 16, 2001
Definitive Test 454A-146A:			
Test Substance Preparation	August 13, 2001	August 13, 2001	August 15, 2001
Matrix Fortifications	August 13, 2001	August 13, 2001	August 15, 2001
Observations and Analytical Sampling	August 17, 2001	August 17, 2001	August 23, 2001
Analytical Data and Draft Report	October 4 – 5, 2001	October 5, 2001	October 8, 2001
Biological Data and Draft Report	October 3 – 5, 2001	October 5, 2001	October 9, 2001
Final Report	January 7, 2002	January 7, 2002	January 7, 2002

Kimberly A. Hoxter

Kimberly A. Hoxter
Quality Assurance Representative

1-7-02

Date

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: Perfluorooctanesulfonate, Potassium Salt (PFOS): A 96-Hour Static-Renewal Acute Toxicity Test with the Sheepshead Minnow (*Cyprinodon variegatus*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 454A-146A

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Susan J. Palmer
Senior Biologist

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07 January 2002
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TABLE OF CONTENTS

TABLE OF CONTENTS

- Continued -

TABLES AND FIGURES

APPENDICES

SUMMARY

SPONSOR:	3M Corporation
SPONSOR'S REPRESENTATIVE:	Ms. Rochelle Robideau
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International, Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER:	454A-146A
TEST SUBSTANCE:	Perfluorooctanesulfonate, Potassium Salt (PFOS)
STUDY:	Perfluorooctanesulfonate, Potassium Salt (PFOS): A 96-Hour Static-Renewal Acute Toxicity Test with the Sheepshead Minnow (*Cyprinodon variegatus*)
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, Solvent Control and 15 mg a.i./L
TEST DATES:	Experimental Start (OECD) – July 6, 2001 Experimental Start (EPA) – August 13, 2001 Biological Termination – August 17, 2001 Experimental Termination – August 17, 2001
LENGTH OF TEST:	96 Hours

TEST ORGANISM:	Sheepshead Minnow (*Cyprinodon variegatus*)	
SOURCE OF TEST ORGANISMS:	Aquatic BioSystems, Inc. Fort Collins, Colorado 80524	
AGE OF TEST ORGANISMS:	Juveniles	
MEASUREMENTS OF 10 NEGATIVE CONTROL FISH:		
WET WEIGHT:	Mean = 0.44 g	Range = 0.21 to 0.66 g
TOTAL LENGTH:	Mean = 3.0 cm	Range = 2.4 to 3.5 cm

96-HOUR LC50:	>15 mg a.i./L
NO MORTALITY CONCENTRATION:	15 mg a.i./L
NO-OBSERVED-EFFECT-CONCENTRATION:	<15 mg a.i./L

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for 3M Corporation at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. An initial trial of the study was conducted under static conditions from July 10, 2001 to July 14, 2001. However, the analytical results were ≤65% of the nominal concentration during the test. Therefore, the test was repeated under static-renewal test conditions. The in-life phase of the definitive test was conducted from August 13, 2001 to August 17, 2001. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 454A-146A in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the acute effects of Perfluorooctanesulfonate, Potassium Salt (PFOS) to the sheepshead minnow, *Cyprinodon variegatus*, during a 96-hour exposure period under static-renewal test conditions.

EXPERIMENTAL DESIGN

Sheepshead minnows were exposed to a single limit concentration, a negative (dilution water) control and a solvent control (0.5 mL methanol/L) for 96 hours, with renewal of test solutions approximately every 24 hours. Three replicate test chambers were maintained in each treatment and control group, with 10 sheepshead minnows in each test chamber for a total of 30 fish per test concentration. Two abiotic replicate test chambers also were maintained at the limit concentration. The nominal test concentration was selected in consultation with the Sponsor, and was based upon the results of exploratory range finding toxicity tests, and the solubility of the test substance in saltwater. The nominal test concentration selected was 20 mg active ingredient (a.i.)/L. The mean measured test concentration was determined from samples of test water collected from each treatment and the control group at the beginning of the test, from new and old test solution at approximately 24, 48 and 72 hours of the test, and from old test solution at test termination.

Sheepshead minnows were indiscriminately assigned to exposure chambers at test initiation, and were transferred daily to freshly prepared test solutions. Observations of mortality and other clinical signs of toxicity were made at approximately 2.5, 24, 48, 72 and 96 hours after test initiation. Cumulative percent mortality observed in the treatment group was used to estimate LC50 values at 2.5, 24, 48, 72 and 96 hours. The no mortality concentration and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and clinical observation data.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "PFOS: A 96-Hour Static Acute Toxicity Test with the Sheepshead Minnow (*Cyprinodon variegatus*)". The protocol was based on procedures outlined in the U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines (draft), OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine* (1); U.S. Environmental Protection Agency, Standard Evaluation Procedure, *Acute Toxicity Test for Estuarine and Marine Organisms* (2); and ASTM Standard E729-88a, *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance was received from 3M Corporation on October 29, 1998 and was assigned Wildlife International, Ltd. identification number 4675. The test substance was described as a white powder. It was identified as FC-95 from lot number 217 (T-6295). Information provided by the Sponsor indicated a purity of 86.9% and an expiration date of August 31, 2006. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

The nominal test concentration was 20 mg a.i./L, based on a test substance purity of 86.9%. On each of Days 0, 1, 2 and 3 of the test, a 250-mL primary stock solution was prepared in methanol at a concentration of 40 mg a.i./mL. The primary stock solution was sonicated for approximately 20 minutes and inverted to mix. Five replicates of the test solution (three biotic and two abiotic replicates) were prepared at a concentration of 20 mg a.i./L by adding the appropriate volume of primary stock to dilution water in the test aquaria to achieve a final volume of 15 L. Each solution was stirred with a stainless steel whisk for approximately one minute. All test solutions appeared clear and colorless. Three solvent control replicates were prepared by adding the appropriate volume of methanol to dilution water in the aquaria. The solvent concentration in the treatment and solvent control groups was 0.5 mL/L.

Test Organism

The sheepshead minnow, *Cyprinodon variegatus*, was selected as the test species for this study. The sheepshead minnow is representative of an important group of aquatic vertebrates and was selected for use in the test based upon past history of use in the laboratory. Sheepshead minnows used in the test were obtained from Aquatic BioSystems, Inc., Fort Collins, Colorado. Identification of the species was verified by the supplier.

The fish were held for approximately seven weeks prior to the test in water from the same source and at approximately the same temperature as used during the test. During the 14-day holding period preceding the test, water temperatures ranged from 22.5 to 22.8°C. The pH of the water ranged from 7.9 to 8.1, salinity was 20‰ (parts per thousand) and dissolved oxygen ranged from 7.0 to 7.2 mg/L (≥89% of saturation). Instrumentation and procedures used for water measurements are described in the *Environmental Conditions* section of this report. The fish were acclimated to test conditions for approximately 51 hours prior to test initiation. During the acclimation period, no mortalities occurred and the fish showed no signs of disease or stress. At test initiation, the fish were collected from the acclimation tank and indiscriminately distributed two at a time to the test chambers until each contained 10 fish.

During the holding period, the fish were fed a commercially-prepared diet (Zeigler Brothers, Inc., Gardners, PA) , as well as *Artemia* nauplii (Summit Artemia, Ogden, Utah). The fish were not fed during the acclimation period (at least two days prior to the test) or during the test.

All fish used in the test were from the same source and year class, and the length of the longest fish was no more than twice the length of the shortest. The average total length of 10 negative control fish measured at the end of the test was 3.0 cm with a range of 2.4 to 3.5 cm. The average wet weight (blotted dry) of 10 negative control fish at the end of the test was 0.44 g with a range of 0.21 to 0.66 g. Loading was 0.30 g fish/L of test water.

Test Apparatus

Test chambers were 25-L polyethylene aquaria containing 15 L of test solution. The depth of water in a representative test chamber was approximately 17.1 cm. Test chambers were positioned in an environmental chamber set to maintain the desired temperature throughout the test. The test chambers were labeled with the project number, test concentration and replicate.

Dilution Water

The water used for culturing and testing was natural seawater collected at Indian River Inlet, Delaware that was filtered and diluted to a salinity of approximately 20‰ with well water. Salinity and pH measurements taken during the four-week period immediately preceding the test are presented in Appendix 1.

The freshly-collected seawater was passed through a sand filter to remove particles greater than approximately 25 µm, and pumped into a 37,800-L storage tank. The filtered saltwater then was diluted with freshwater from a well on the Wildlife International, Ltd. site and aerated with spray nozzles. The 20‰ water was filtered to 0.45 µm to remove microorganisms and fine particles prior to its use in the test. The results of periodic analyses performed to measure the concentrations of selected contaminants in the saltwater used by Wildlife International, Ltd. are presented in Appendix 2.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding, acclimation and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was approximately 139 lux at the surface of the water of one representative test chamber.

Temperature was measured in each test chamber at the beginning of the test, prior to and after test solution renewal, and at test termination using a liquid-in-glass thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder, which was verified prior to test initiation with a liquid-in-glass thermometer. The target test temperature during the study was 22 ± 2°C. Dissolved oxygen and pH measurements were made on water samples collected from all replicate test chambers of each treatment and control at test initiation, prior to and after test solution renewal, and at test termination. Salinity was measured in the dilution water prior to test initiation and at test termination.

Light intensity was measured using a SPER Scientific Ltd. Model 840006C light meter. Measurements of pH were made using a Fisher Accumet Model 915 pH meter, and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter. Salinity was measured using a Bio-Marine, Inc. Aquafauna refractometer.

Observations

All organisms were observed periodically to determine the number of mortalities in each control and treatment group. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 2.5, 24, 48, 72 and 96 hours after test initiation.

Statistical Analyses

The use of a single test concentration precluded the statistical calculation of LC50 values. Therefore, since there were no mortalities during the study, the 2.5, 24, 48, 72 and 96-hour LC50 values were estimated to be greater than the single concentration tested. The no mortality concentration and NOEC were determined by visual interpretation of the mortality and observation data.

Analytical Chemistry

Water samples were collected at mid-depth from each biotic test chamber of each treatment and control group at the beginning of the test, prior to and after test solution renewal at approximately 24, 48 and 72 hours of the test, and at test termination to measure concentrations of the test substance. Samples also were collected for analysis from the abiotic test chambers prepared at 20 mg a.i./L prior to and after test solution renewal at approximately 24, 48 and 72 hours, and at test termination. All samples were collected in plastic vials and analyzed as soon as possible without storage. Analytical procedures used in the analysis of the samples are provided in Appendix 3.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of PFOS in water samples collected during the test are presented in Table 1 and in the analytical chemistry report (Appendix 3). The single nominal concentration selected for use in this study was 20 mg a.i./L. Samples collected from new test solutions at test initiation and at approximately 24, 48 and 72 hours had measured concentrations that ranged from 74.9 to 92.9% of nominal concentrations. Samples collected from old test solutions at approximately 24, 48 and 72 hours and at test termination had measured concentrations that ranged from 55.6 to 89.9% of nominal concentrations. The abiotic 20 mg a.i./L samples had measured concentrations that ranged from 79.4 to 88.5% of nominal in the new test solutions, and from 45.0 to 54.4% of nominal in the old test solutions. The measured concentrations of PFOS in the abiotic samples from the old solutions were

slightly lower than the concentrations of the 20 mg a.i./L treatment group samples with the fish present. The most plausible reason for this was increased deposition of test substance at the limit of solubility in the absence of the natural mixing action provided by the movement of the fish. When measured concentrations of the 20 mg a.i./L biotic samples analyzed at each sampling interval were averaged, the mean measured concentration for this study was 15 mg a.i./L, representing 75% of the nominal concentration. The results of the study were based on the mean measured concentration.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH are presented in Table 2. Temperatures were within the 22 ± 2°C range established for the test. Dissolved oxygen concentrations were ≥7.0 mg/L (89% of saturation) in the new test solutions and ranged from 1.6 to 6.7 mg/L (20 to 86% of saturation) in the old test solutions. Although dissolved oxygen concentrations dropped below 60% of saturation several times during the test, the biological results of the study indicate that this did not have an adverse effect on the results of the study. Measurements of pH ranged from 7.9 to 8.3 during the test. Salinity measurements of the dilution water at test initiation and termination were 20‰ (Table 2).

Daily observations of mortality and other signs of toxicity observed during the test are presented in Table 3. Sheepshead minnows in the negative control and solvent control groups appeared normal and healthy throughout the test period. No mortalities occurred in the 15 mg a.i./L treatment group during the test. However, upon transfer to new test solution at approximately 48 and 72 hours, some of the fish were observed swimming erratically and turning a dark color. The fish appeared normal within approximately two hours, although one fish in the 15 mg a.i./L treatment group appeared discolored at test termination. LC50 values estimated from the mortality data at 2.5, 24, 48, 72 and 96 hours are shown in Table 4.

CONCLUSIONS

The 96-hour LC50 value for the sheepshead minnow, *Cyprinodon variegatus*, exposed to Perfluorooctanesulfonate, Potassium Salt (PFOS) was >15 mg a.i./L, the single concentration tested. The 96-hour no mortality concentration was 15 mg a.i./L. Based on the transitory effects observed in the 15 mg a.i./L treatment group after transfer to fresh test solutions, the NOEC was <15 mg a.i./L.

REFERENCES

1 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine.*

2 **U.S. Environmental Protection Agency.** 1985. *Standard Evaluation Procedure, Acute Toxicity Test for Estuarine and Maine Organisms (Estuarine Fish 96-Hour Acute Toxicity Test).* Hazard Evaluation Division. Office of Pesticide Programs. EPA-540/9-85-009. Washington, DC.

3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.

Table 1

Summary of Analytical Chemistry Data

Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)[1]	Percent of Nominal[2]	Mean Measured Concentration (mg a.i./L)	Mean Measured Percent of Nominal
Negative Control	A	0	<LOQ	--	<LOQ	--
	B	0	<LOQ	--		
	C	0	<LOQ	--		
	A	24 (Old)	<LOQ	--		
	B	24 (Old)	<LOQ	--		
	C	24 (Old)	<LOQ	--		
	A	24 (New)	<LOQ	--		
	B	24 (New)	<LOQ	--		
	C	24 (New)	<LOQ	--		
	A	48 (Old)	<LOQ	--		
	B	48 (Old)	<LOQ	--		
	C	48 (Old)	<LOQ	--		
	A	48 (New)	<LOQ	--		
	B	48 (New)	<LOQ	--		
	C	48 (New)	<LOQ	--		
	A	72 (Old)	<LOQ	--		
	B	72 (Old)	<LOQ	--		
	C	72 (Old)	<LOQ	--		
	A	72 (New)	<LOQ	--		
	B	72 (New)	<LOQ	--		
	C	72 (New)	<LOQ	--		
	A	96	<LOQ	--		
	B	96	<LOQ	--		
	C	96	<LOQ	--		
Solvent Control	A	0	<LOQ	--	<LOQ	--
	B	0	<LOQ	--		
	C	0	<LOQ	--		
	A	24 (Old)	<LOQ	--		
	B	24 (Old)	<LOQ	--		
	C	24 (Old)	<LOQ	--		
	A	24 (New)	<LOQ	--		
	B	24 (New)	<LOQ	--		
	C	24 (New)	<LOQ	--		
	A	48 (Old)	<LOQ	--		
	B	48 (Old)	<LOQ	--		
	C	48 (Old)	<LOQ	--		
	A	48 (New)	<LOQ	--		
	B	48 (New)	<LOQ	--		
	C	48 (New)	<LOQ	--		
	A	72 (Old)	<LOQ	--		
	B	72 (Old)	<LOQ	--		
	C	72 (Old)	<LOQ	--		
	A	72 (New)	<LOQ	--		
	B	72 (New)	<LOQ	--		
	C	72 (New)	<LOQ	--		
	A	96	<LOQ	--		
	B	96	<LOQ	--		
	C	96	<LOQ	--		

[1] The limit of quantitation (LOQ) was 5.00 mg a.i./L.
[2] Results generated using MacQuan Version 1.6 software in full precision mode. Manual calculations may differ slightly.

Table 1 (Continued)

Summary of Analytical Chemistry Data

Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)[1]	Percent of Nominal[2]	Mean Measured Concentration (mg a.i./L)	Mean Measured Percent of Nominal
20	A	0	16.4	82.0	15	75
	B	0	15.7	78.6		
	C	0	16.0	80.0		
	A	24 (Old)	15.2	76.1		
	B	24 (Old)	13.3	66.6		
	C	24 (Old)	15.2	76.2		
	A	24 (New)	16.2	81.2		
	B	24 (New)	15.7	78.7		
	C	24 (New)	15.0	74.9		
	A	48 (Old)	13.4	66.9		
	B	48 (Old)	11.3	56.4		
	C	48 (Old)	12.7	63.4		
	A	48 (New)	17.4	86.9		
	B	48 (New)	18.2	90.9		
	C	48 (New)	18.6	92.9		
	A	72 (Old)	15.2	75.8		
	B	72 (Old)	18.0	89.9		
	C	72 (Old)	16.2	80.9		
	A	72 (New)	17.0	85.2		
	B	72 (New)	16.6	82.9		
	C	72 (New)	17.4	87.1		
	A	96	13.7	68.4		
	B	96	11.1	55.6		
	C	96	13.4	67.1		
20 (Abiotic)	D	24 (Old)	10.9	54.4	13	65
	E	24 (Old)	9.22	46.1		
	D	24 (New)	16.1	80.3		
	E	24 (New)	16.6	83.0		
	D	48 (Old)	9.62	48.1		
	E	48 (Old)	9.69	48.5		
	D	48 (New)	16.6	83.2		
	E	48 (New)	17.5	87.3		
	D	72 (Old)	9.01	45.0		
	E	72 (Old)	9.25	46.3		
	D	72 (New)	17.7	88.5		
	E	72 (New)	15.9	79.4		
	D	96	9.84	49.2		
	E	96	9.22	46.1		

[1] The limit of quantitation (LOQ) was 5.00 mg a.i./L.

[2] Results generated using MacQuan version 1.6 software in full precision mode. Manual calculations may differ slightly.

Table 2

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Mean Measured Concentration (mg a.i./L)	Replicate	0 Hour [1]			24 Hours (Old)			24 Hours (New)			48 Hours (Old)		
		Temp [2] (°C)	DO [3] (mg/L)	pH	Temp (°C)	DO (mg/L)	pH	Temp (°C)	DO (mg/L)	pH	Temp (°C)	DO (mg/L)	pH
Negative Control	A	22.2	7.0	8.2	22.4	6.2	8.1	22.6	7.1	8.2	22.0	5.3	8.0
	B	22.3	7.1	8.2	22.4	6.0	8.1	22.6	7.2	8.2	22.1	4.6	7.9
	C	22.4	7.1	8.3	22.6	5.9	8.2	22.5	7.2	8.2	22.1	3.4	8.0
Solvent Control	A	22.5	7.1	8.3	22.8	5.8	8.1	22.6	7.3	8.3	22.3	4.4	8.0
	B	22.5	7.1	8.3	22.8	5.9	8.2	22.6	7.3	8.3	22.4	3.7	8.0
	C	22.6	7.1	8.3	22.9	5.9	8.2	22.7	7.3	8.3	22.4	3.6	8.0
15	A	22.7	7.1	8.3	23.0	5.6	8.1	22.6	7.3	8.3	22.5	4.6	8.0
	B	22.8	7.1	8.3	23.1	5.6	8.1	22.7	7.3	8.3	22.5	3.0	8.0
	C	22.8	7.1	8.3	23.1	5.4	8.1	22.7	7.3	8.3	22.6	5.0	8.1
	D [4]	22.7	7.1	8.3	23.1	6.7	8.3	22.8	7.3	8.3	22.7	5.0	8.1
	E [4]	22.8	7.1	8.3	23.1	5.2	8.2	22.7	7.3	8.3	22.7	4.8	8.1

[1] The 0 and 96-hour dilution water measurements for salinity were 20‰.
[2] Temperature measured continuously during the test remained between 21.5 and 22.5°C.
[3] A dissolved oxygen concentration of 4.7 mg/L represents 60% saturation at 22°C in saltwater with a salinity of 20‰.
[4] Abiotic replicates.

Table 2 (Continued)

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Mean Measured Concentration (mg a.i./L)	Replicate	48 Hours (New)			72 Hours (Old)			72 Hours (New)			96 Hours (Old)[1]		
		Temp[2] (°C)	DO[3] (mg/L)	pH	Temp (°C)	DO (mg/L)	pH	Temp (°C)	DO (mg/L)	pH	Temp (°C)	DO (mg/L)	pH
Negative Control	A	22.1	7.2	8.2	22.2	5.5	8.0	22.1	7.4	8.2	22.1	3.8	7.9
	B	22.1	7.3	8.2	22.3	3.9	7.9	22.0	7.4	8.2	21.9	3.5	7.9
	C	22.2	7.3	8.2	22.3	3.7	8.0	22.1	7.4	8.2	22.1	2.8	7.9
Solvent Control	A	22.3	7.2	8.2	22.3	4.0	8.0	22.2	7.4	8.2	22.1	2.6	7.9
	B	22.4	7.2	8.2	22.4	3.0	7.9	22.2	7.5	8.2	22.2	2.7	7.9
	C	22.4	7.2	8.2	22.4	4.0	8.0	22.3	7.6	8.2	22.3	1.7	7.9
15	A	22.4	7.2	8.2	22.5	3.6	7.9	22.2	7.6	8.2	22.4	1.9	7.9
	B	22.5	7.2	8.2	22.5	3.6	8.0	22.2	7.5	8.2	22.3	1.6	7.9
	C	22.5	7.2	8.2	22.6	3.3	7.9	22.3	7.4	8.2	22.4	3.3	7.9
	D[4]	22.6	7.4	8.3	22.6	5.4	8.1	22.3	7.5	8.2	22.5	3.4	8.0
	E[4]	22.5	7.4	8.3	22.6	5.5	8.1	22.3	7.5	8.2	22.6	3.2	8.0

[1] The 0 and 96-hour dilution water measurements for salinity were 20‰.
[2] Temperature measured continuously during the test remained between 21.5 and 22.5°C.
[3] A dissolved oxygen concentration of 4.7 mg/L represents 60% saturation at 22°C in saltwater with a salinity of 20‰.
[4] Abiotic replicates.

Table 3

Cumulative Percent Mortality and Treatment-Related Effects

Mean Measured Concentration (mg a.i./L)	Rep.	No. Exposed	2.5 Hours		24 Hours[1]		48 Hours[1]		72 Hours[1]		96 Hours		Cumulative Percent Mortality
			No. Dead[2]	Observed Effects[3]	No. Dead	Observed Effects	No. Dead	Observed Effects	No. Dead	Observed Effects	No. Dead	Observed Effects	
Negative Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
	C	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
Solvent Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
	C	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
15	A	10	0	10 AN	0	10 AN	0	2E,D; 8AN	0	3E,D; 8AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	3E,D; 1R; 6AN	0	4E,D; 6AN	0	10 AN	
	C	10	0	10 AN	0	10 AN	0	1E,D; 9AN	0	2E,D; 9AN	0	1D; 9AN	

[1] All fish were transferred to fresh test solution at approximately 24, 48 and 72 hours.
[2] Cumulative number of dead fish.
[3] Observed Effects: AN = appear normal; E = erratic swimming; D = discoloration (dark); R = lying on bottom.

Table 4

LC50 Values

Time	LC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	Statistical Method
2.5 Hours	>15	--[1]	NA[2]
24 Hours	>15	--[1]	NA[2]
48 Hours	>15	--[1]	NA[2]
72 Hours	>15	--[1]	NA[2]
96 Hours	>15	--[1]	NA[2]

[1] 95% confidence limits could not be calculated from the data.
[2] NA = not applicable; LC50 value was estimated by visual interpretation of the mortality data.

Appendix 1

Salinity and pH of Saltwater Measured During the
4-Week Period Immediately Preceding the Test

Parameter	Mean	Range
Salinity (‰)	20 (n = 4)	20 – 20
pH	8.2 (n = 4)	8.1 – 8.3

Appendix 2

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Saltwater[1]

Pesticides And Organics			
Component	Measured Concentration	Component	Measured Concentration
Aclonifen	<0.03 µg/L	Dichlorvos	<0.01 µg/L
Alachlor	<0.01 µg/L	Dicofol	<0.25 µg/L
Ametryn	<0.01 µg/L	Diethyltoluamide	<0.02 µg/L
Atrazine	<0.01 µg/L	Difenoconazole	<0.03 µg/L
Azinphos-ethyl	<0.04 µg/L	Dimethoate	<0.02 µg/L
Azinphos-methyl	<0.08 µg/L	Dimethomorph	<0.05 µg/L
Azoxystrobin	<0.25 µg/L	Disulfoton	<0.02 µg/L
Bifenthrin	<0.05 µg/L	DMST	<0.05 µg/L
Bioallethrin	<0.05 µg/L	Dodemorph	<0.01 µg/L
Bitertanol	<0.05 µg/L	Endosulfan-α	<0.01 µg/L
Bromacil	<0.05 µg/L	Endosulfan-β	<0.01 µg/L
Bromophos	<0.02 µg/L	Endosulfan-sulfate	<0.02 µg/L
Bromophos-ethyl	<0.02 µg/L	Epoxiconazole	<0.05 µg/L
Bromopropylate	<0.02 µg/L	Eptam	<0.02 µg/L
Bupirimate	<0.05 µg/L	Esfenvalerate	<0.02 µg/L
Carbaryl	<0.05 µg/L	Ethion	<0.05 µg/L
Carbofuran	<0.03 µg/L	Ethofumesate	<0.02 µg/L
Carboxin	<0.02 µg/L	Ethoprophos	<0.01 µg/L
Chlorfenvinphos	<0.02 µg/L	Etridiazole	<0.02 µg/L
Chloridazon	<0.05 µg/L	Etrimfos	<0.05 µg/L
Chlorpropham	<0.02 µg/L	Fenarimol	<0.05 µg/L
Chlorpyrifos	<0.01 µg/L	Fenchlorphos	<0.01 µg/L
Chlorpyrifos-methyl	<0.01 µg/L	Fenitrothion	<0.03 µg/L
Chlorothalonil	<0.04 µg/L	Fenoxycarb	<0.03 µg/L
Coumaphos	<0.02 µg/L	Fenpiclonil	<0.05 µg/L
Cyanazine	<0.05 µg/L	Fenpropathrin	<0.25 µg/L
Cyfluthrin	<0.05 µg/L	Fenpropimorph	<0.01 µg/L
Cypermethrin	<0.25 µg/L	Fenthion	<0.01 µg/L
Cyproconazole	<0.05 µg/L	Fenvalerate	<0.02 µg/L
Deltamethrin	<0.02 µg/L	Fluazifop-butyl	<0.02 µg/L
Demeton	<0.02 µg/L	Fluoroglycofen-ethyl	<0.02 µg/L
Demeton-O	<0.02 µg/L	Fluroxypyr-meptyl	<0.05 µg/L
Desethylatrazine	<0.01 µg/L	Flutolanil	<0.02 µg/L
Desisopropylatrazine	<0.02 µg/L	Fonophos	<0.01 µg/L
Desmetryn	<0.01 µg/L	Furalaxyl	<0.02 µg/L
Diazinon	<0.01 µg/L	Heptenophos	<0.02 µg/L
Dichlobenil	<0.01 µg/L	Imazalil	<0.01 µg/L
Dichloran	<0.03 µg/L	Iprodion	<0.05 µg/L
Dichlorbenzamide	<0.02 µg/L	Kresoxim-methyl	<0.02 µg/L
Dichlorfenthion	<0.01 µg/L	Lenacil	<0.05 µg/L
Dichlorfluanid	<0.03 µg/L	Lindane	<0.02 µg/L

[1]Analyses performed by TNO Nutrition and Food Institute on samples collected on November 15, 2000.

Appendix 2 (Continued)

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Saltwater[1]

Pesticides And Organics (Page 2)			
Component	Measured Concentration	Component	Measured Concentration
Malathion	<0.02 µg/L	Methoxychlor	<0.01 µg/L
Metalaxyl	<0.05 µg/L	Metolachlor	<0.01 µg/L
Metamitron	<0.05 µg/L	Metribuzin	<0.02 µg/L
Metazachlor	<0.02 µg/L	Mevinphos	<0.01 µg/L
Methidathion	<0.02 µg/L	Nitrothal-Isopropyl	<0.05 µg/L
Paclobutazole	<0.05 µg/L	Pyrifenox-1	<0.01 µg/L
Parathion	<0.01 µg/L	Pyrifenox-2	<0.01 µg/L
Parathion-methyl	<0.01 µg/L	Pyrimethanil	<0.01 µg/L
Penconazole	<0.05 µg/L	Quizalofop-ethyl	<0.02 µg/L
Pendimethalin	<0.03 µg/L	Simazine	<0.01 µg/L
Permethrin-cis	<0.01 µg/L	Sulfotep	<0.02 µg/L
Permethrin-trans	<0.01 µg/L	Tebuconazole	<0.05 µg/L
Phosalone	<0.05 µg/L	Tebufenpyrad	<0.05 µg/L
Phosmet	<0.02 µg/L	Terbutryn	<0.01 µg/L
Phosphamidon-cis	<0.05 µg/L	Terbuthylazine	<0.01 µg/L
Pirimicarb	<0.01 µg/L	Tetrachlorvinphos	<0.01 µg/L
Pirimiphos-ethyl	<0.01 µg/L	Tetrahydroftalimide	<0.05 µg/L
Pirimiphos-methyl	<0.01 µg/L	Tetramethrin	<0.01 µg/L
Prochloraz	<0.02 µg/L	Thiabendazole	<0.05 µg/L
Procymidon	<0.01 µg/L	Thiometon	<0.04 µg/L
Prometryn	<0.01 µg/L	Tolclofos-methyl	<0.01 µg/L
Propachlor	<0.01 µg/L	Tolylfluanid	<0.04 µg/L
Propazine	<0.01 µg/L	Triadimefon	<0.05 µg/L
Propham	<0.02 µg/L	Triadimenol	<0.05 µg/L
Propiconazole	<0.05 µg/L	Triallate	<0.02 µg/L
Propoxur	<0.03 µg/L	Triazophos	<0.02 µg/L
Propyzamide	<0.02 µg/L	Trifluralin	<0.02 µg/L
Prosulfocarb	<0.02 µg/L	Vamidothion	<0.01 µg/L
Pyrazophos	<0.03 µg/L	Vinclozolin	<0.01 µg/L
Metals			
Magnesium	730 mg/L	Nickel	<14 µg/L
Sodium	5,800 mg/L	Copper	<10.0 µg/L
Calcium	270 mg/L	Zinc	<20.0 µg/L
Iron	<0.03 mg/L	Molybdenum	<7.0 µg/L
Potassium	200 mg/L	Silver	<3.0 µg/L
Aluminum	<0.18 mg/L	Cadmium	<3.0 µg/L
Manganese	<1.0 µg/L	Arsenic	1.2 µg/L
Beryllium	<3.0 µg/L	Mercury	<0.025 µg/L
Chromium	<6.0 µg/L	Selenium	<1 µg/L
Cobalt	<4.0 µg/L		

[1]Analyses performed by TNO Nutrition and Food Institute on samples collected on November 15, 2000.

Appendix 3

THE ANALYSIS OF PFOS IN FILTERED SALTWATER

IN SUPPORT OF

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454A-146A

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static-Renewal Acute Toxicity Test with the Sheepshead Minnow (*Cyprinodon variegatus*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NO.: 454A-146A

PRINCIPAL INVESTIGATOR:

Raymond L. Van Hoven, Ph.D.
Scientist

01/07/02
DATE

MANAGEMENT:

Willard B. Nixon, Ph.D.
Director, Analytical Chemistry

1/7/02
DATE

Introduction

Filtered saltwater samples were collected from a 96-hour static-renewal acute toxicity test designed to determine the effects of PFOS (Perfluorooctanesulfonate, Potassium Salt) to the sheepshead minnow (*Cyprinodon variegatus*). This study was conducted by Wildlife International, Ltd. and identified as Project No.: 454A-146A. The analyses of these water samples were performed at Wildlife International, Ltd. using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). Samples were received for analysis on August 13, 14, 15, 16 and 17, 2001 and were analyzed on each sample receipt day.

Test Substance and Internal Standard

The test substance used for this study was Wildlife International, Ltd. identification number 4675. The test substance, referred to hereafter as PFOS, was used to prepare calibration standards and matrix fortification samples.

The internal standard was received from 3M Corporation on July 2, 1998 and was assigned Wildlife International, Ltd. identification number 4526 upon receipt. The internal standard, a granular material, was identified as: 1H, 1H, 2H, 2H Perfluorooctane Sulfonic Acid, Chemical Abstract Number: 27619-97-2. The standard, referred to hereafter as 4H PFOS, was stored under ambient conditions.

Analytical Method

The method used for the analysis of the filtered saltwater samples was developed at Wildlife International, Ltd. and entitled "Analytical Method for the Determination of PFOS in Freshwater, Saltwater, and Algal Medium". This methodology was included as Appendix II of Wildlife International, Ltd. protocol number 454/011299/MVAL/SUB454. It was based upon methodology provided by 3M Corporation. Several modifications from the validated methodology were implemented for the present study. First, the concentration of the internal standard was changed from 100 µg/L to 10.0 µg/L to better match the calibration and test sample instrumental concentrations. Second, a guard cartridge was used in conjunction with a shorter (50 mm vs. 100 mm) analytical column for long-term protection of the analytical column and faster run times.

Samples were diluted in a 50% methanol : 50% NANOpure® water solution containing 10.0 µg 4H PFOS /L and 0.05% formic acid (v/v) so that they fell within the calibration range of the PFOS methodology.

Concentrations of the PFOS in the standards and samples were determined by reverse-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph (HPLC) with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C_{18} analytical column (50 mm × 2 mm I.D., 3-µm particle size) fitted with a Keystone Javelin C_{18} guard cartridge (20 x 2 mm). The instrument parameters are summarized in Table 1. A method flowchart is provided in Figure 1.

Calibration Curve and Limit of Quantitation

Calibration standards of PFOS prepared in a 50% methanol : 50% NANOpure® water solution containing 10.0 µg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v), ranging in concentration from 0.500 to 5.00 µg a.i./L, were analyzed with the samples. The same and most prominent peak response for PFOS was utilized to monitor PFOS in all calibration, quality control, and study samples. No attempt was made to quantify PFOS on the basis of individual isomeric components. Linear regression equations were generated using peak area response ratios (PFOS : internal standard) versus the respective concentration ratios (PFOS : internal standard) of the calibration standards. A typical calibration curve is presented in Figure 2. The concentration of PFOS in the samples was determined by substituting the peak area response ratios into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 3 and 4, respectively.

The method limit of quantitation (LOQ) for these analyses was set at 5.00 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.000500 mg a.i./L) and the dilution factor of the matrix blank samples (10,000).

Matrix Blank and Fortification Samples

Five matrix blank samples were analyzed to determine possible interference. No interferences were observed at or above the LOQ during samples analyses (Table 2). A representative ion chromatogram of a matrix blank is presented in Figure 5.

Filtered saltwater was fortified at 18 and 22 mg a.i./L and analyzed concurrently with the samples to determine the mean procedural recovery (Table 3). Sample concentrations were not corrected for the mean procedural recovery of 99.2%. A representative ion chromatogram of a matrix fortification is presented in Figure 6.

Example Calculations

Sample number 454A-146A-9, nominal concentration of 20 mg a.i./L in filtered saltwater.

Peak Area Ratio = Analyte Peak Area/Internal Standard Peak Area

Concentration Ratio = Concentration of Analyte/Concentration of Internal Standard

Internal Standard Concentration: 0.0100 mg/L

First Initial Volume: 0.100 mL

First Final Volume: 10.0 mL

Second Initial Volume: 0.100 mL

Second Final Volume: 10.0 mL

Dilution Factor: 10,000

PFOS Peak Area: 252985

Internal Standard Peak Area: 469623

Peak Area Ratio: 0.5387

Calibration curve equation.

Slope: 3.2154

Intercept: 0.0244

Curve is weighted (1/x)

$$\text{PFOS (mg a.i./L) at instrument} = \frac{\text{Peak area ratio - (Y-intercept)}}{\text{Slope}} \times \text{Internal Standard Concentration}$$

$$= \frac{0.5387 - 0.0244}{3.2154} \times 0.0100$$

$$= 0.001599$$

PFOS (mg a.i./L) in sample = PFOS (mg a.i./L) at instrument × Dilution Factor

$$= 0.001599 \times 10{,}000$$

$$= 16.0$$

$$\text{Percent of Nominal Concentration} = \frac{\text{PFOS (mg a.i./L) in sample}}{\text{PFOS (mg a.i./L) nominal}} \times 100$$

$$= \frac{16.0}{20} \times 100$$

$$= 80.0\%$$

Quantitation software for recoveries: MacQuan, version 1.6.

RESULTS

Sample Analysis

Filtered saltwater samples were collected from a 96-hour static-renewal acute toxicity test with the sheepshead minnow (*Cyprinodon variegatus*). New solutions were collected at test initiation, August 13, 2001 (Hour 0). New and old solutions were collected on August 14 (Hour 24), August 15 (Hour 48), and August 16 (Hour 72), 2001. Old solutions were collected at test termination, August 17, 2001 (Hour 96).

The measured concentrations of PFOS in the samples collected from the 20 mg a.i./L treatment group at initiation of exposure of the test organisms (Hour 0) had measured concentrations ranging from 15.7 to 16.4 mg a.i./L, corresponding to 78.6 to 82.0% of the nominal concentrations (Table 4). New samples collected at Hours 24, 48, and 72 had measured concentrations ranging from 15.0 to 16.2 mg a.i./L, 17.4 to 18.6 mg a.i./L, and 16.6 to 17.4 mg a.i./L, respectively. These values corresponded to percent of nominal ranging from 74.9 to 81.2%, 86.9 to 92.9%, and 82.9 to 87.1%, respectively. Old samples collected at Hours 24, 48, and 72 had measured concentrations ranging from 13.3 to 15.2 mg a.i./L, 11.3 to 13.4 mg a.i./L, and 15.2 to 18.0 mg a.i./L, respectively. These values corresponded to percent of nominal ranging from 66.6 to 76.2%, 56.4 to 66.9%, and 75.8 to 89.9%, respectively. The

measured concentrations of PFOS in the samples collected at test termination (Hour 96) had measured concentrations ranging from 11.1 to 13.7 mg a.i./L, corresponding to 55.6 to 68.4% of the nominal concentrations.

The measured concentrations of PFOS from the abiotic 20 mg a.i./L treatment group were slightly lower (mean measured concentration = 13 mg a.i./L) than those from the 20 mg a.i./L treatment group (mean measured concentration = 15 mg a.i./L) with the fish present (Table 4). The most plausible reason for this was increased deposition of test substance at the limit of solubility in the absence of the natural mixing action provided by the movement of the fish. A representative ion chromatogram of a test sample is shown in Figure 7.

Table 1

Typical HPLC/MS Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer API 100LC Mass Spectrometer (HPLC/MS) equipped with a Perkin-Elmer TurboIonSpray ion source. Operated in selective ion monitoring mode (SIM).
ANALYTICAL COLUMN:	Keystone Betasil C_{18} column (50 mm × 2 mm I.D., 3-µm particle size)
GUARD COLUMN:	Keystone Javelin C_{18} column (20 x 2 mm)
OVEN TEMPERATURE:	30°C
STOP TIME:	5.00 minutes
FLOW RATE:	0.220 mL/minute
MOBILE PHASE:	72.0% Methanol : 28.0% NANOpure® water containing 0.1% Formic Acid
INJECTION VOLUME:	25.0 µL
PFOS RETENTION TIME:	Approximately 4.4 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 3.0 minutes
PFOS MONITORED MASS:	499 amu
INTERNAL STANDARD MONITORED MASS:	427 amu

Table 2

Matrix Blanks Analyzed Concurrently During Sample Analysis

Sample		Measured Concentration of PFOS[1] (mg a.i./L)
Number (454A-146A-)	Type	
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ
MAB-3	Matrix Blank	< LOQ
MAB-4	Matrix Blank	< LOQ
MAB-5	Matrix Blank	< LOQ

[1] The limit of quantitation (LOQ) was 5.00 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.000500 mg a.i./L) and the dilution factor of the matrix blank samples (10,000).

Table 3

Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-146A-)	Concentrations of PFOS (mg a.i./L)		Percent Recovered[1]
	Fortified[1]	Measured[1]	
MAS-1	18.0	16.2	89.9
MAS-3	18.0	17.9	99.4
MAS-5	18.0	16.6	92.4
MAS-7	18.0	18.4	102
MAS-9	18.0	18.1	101
MAS-2	22.0	20.2	92.0
MAS-4	22.0	22.4	102
MAS-6	22.0	22.0	99.9
MAS-8	22.0	23.8	108
MAS-10	22.0	23.0	105
		Mean	= 99.2
		Standard Deviation	= 5.92
		CV	= 5.97%
		N	= 10

[1] Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

Table 4

Measured Concentrations of PFOS in Filtered Saltwater Samples
from a Sheepshead Minnow (*Cyprinodon variegatus*) 96-Hour Toxicity Test

Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)[1]	Percent of Nominal[2]	Mean Measured Concentration (mg a.i./L)	Mean Measured Percent of Nominal
0.0 (Negative Control)	A	0	<LOQ	--	<LOQ	--
	B	0	<LOQ	--		
	C	0	<LOQ	--		
	A	24 (Old)	<LOQ	--		
	B	24 (Old)	<LOQ	--		
	C	24 (Old)	<LOQ	--		
	A	24 (New)	<LOQ	--		
	B	24 (New)	<LOQ	--		
	C	24 (New)	<LOQ	--		
	A	48 (Old)	<LOQ	--		
	B	48 (Old)	<LOQ	--		
	C	48 (Old)	<LOQ	--		
	A	48 (New)	<LOQ	--		
	B	48 (New)	<LOQ	--		
	C	48 (New)	<LOQ	--		
	A	72 (Old)	<LOQ	--		
	B	72 (Old)	<LOQ	--		
	C	72 (Old)	<LOQ	--		
	A	72 (New)	<LOQ	--		
	B	72 (New)	<LOQ	--		
	C	72 (New)	<LOQ	--		
	A	96	<LOQ	--		
	B	96	<LOQ	--		
	C	96	<LOQ	--		

[1] The limit of quantitation (LOQ) was 5.00 mg a.i./L.

[2] Results were generated using MacQuan version 1.6 software in full precision mode. Manual calculations may differ slightly.

Table 4 (Continued)

Measured Concentrations of PFOS in Filtered Saltwater Samples
from a Sheepshead Minnow (*Cyprinodon variegatus*) 96-Hour Toxicity Test

Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)[1]	Percent of Nominal[2]	Mean Measured Concentration (mg a.i./L)	Mean Measured Percent of Nominal
0.0	A	0	<LOQ	--	<LOQ	--
	B	0	<LOQ	--		
(Solvent Control)	C	0	<LOQ	--		
	A	24 (Old)	<LOQ	--		
	B	24 (Old)	<LOQ	--		
	C	24 (Old)	<LOQ	--		
	A	24 (New)	<LOQ	--		
	B	24 (New)	<LOQ	--		
	C	24 (New)	<LOQ	--		
	A	48 (Old)	<LOQ	--		
	B	48 (Old)	<LOQ	--		
	C	48 (Old)	<LOQ	--		
	A	48 (New)	<LOQ	--		
	B	48 (New)	<LOQ	--		
	C	48 (New)	<LOQ	--		
	A	72 (Old)	<LOQ	--		
	B	72 (Old)	<LOQ	--		
	C	72 (Old)	<LOQ	--		
	A	72 (New)	<LOQ	--		
	B	72 (New)	<LOQ	--		
	C	72 (New)	<LOQ	--		
	A	96	<LOQ	--		
	B	96	<LOQ	--		
	C	96	<LOQ	--		

[1] The limit of quantitation (LOQ) was 5.00 mg a.i./L.
[2] Results were generated using MacQuan version 1.6 software in full precision mode. Manual calculations may differ slightly.

Table 4 (Continued)

Measured Concentrations of PFOS in Filtered Saltwater Samples
from a Sheepshead Minnow (*Cyprinodon variegatus*) 96-Hour Toxicity Test

Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)[1]	Percent of Nominal[2]	Mean Measured Concentration (mg a.i./L)	Mean Measured Percent of Nominal
20	A	0	16.4	82.0	15	75
	B	0	15.7	78.6		
	C	0	16.0	80.0		
	A	24 (Old)	15.2	76.1		
	B	24 (Old)	13.3	66.6		
	C	24 (Old)	15.2	76.2		
	A	24 (New)	16.2	81.2		
	B	24 (New)	15.7	78.7		
	C	24 (New)	15.0	74.9		
	A	48 (Old)	13.4	66.9		
	B	48 (Old)	11.3	56.4		
	C	48 (Old)	12.7	63.4		
	A	48 (New)	17.4	86.9		
	B	48 (New)	18.2	90.9		
	C	48 (New)	18.6	92.9		
	A	72 (Old)	15.2	75.8		
	B	72 (Old)	18.0	89.9		
	C	72 (Old)	16.2	80.9		
	A	72 (New)	17.0	85.2		
	B	72 (New)	16.6	82.9		
	C	72 (New)	17.4	87.1		
	A	96	13.7	68.4		
	B	96	11.1	55.6		
	C	96	13.4	67.1		

[1] The limit of quantitation (LOQ) was 5.00 mg a.i./L.
[2] Results were generated using MacQuan version 1.6 software in full precision mode. Manual calculations may differ slightly.

Table 4 (Continued)

Measured Concentrations of PFOS in Filtered Saltwater Samples
from a Sheepshead Minnow (*Cyprinodon variegatus*) 96-Hour Toxicity Test

Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)[1]	Percent of Nominal[2]	Mean Measured Concentration (mg a.i./L)	Mean Measured Percent of Nominal
20 (Abiotic)	D	24 (Old)	10.9	54.4	13	65
	E	24 (Old)	9.22	46.1		
	D	24 (New)	16.1	80.3		
	E	24 (New)	16.6	83.0		
	D	48 (Old)	9.62	48.1		
	E	48 (Old)	9.69	48.5		
	D	48 (New)	16.6	83.2		
	E	48 (New)	17.5	87.3		
	D	72 (Old)	9.01	45.0		
	E	72 (Old)	9.25	46.3		
	D	72 (New)	17.7	88.5		
	E	72 (New)	15.9	79.4		
	D	96	9.84	49.2		
	E	96	9.22	46.1		

[1] The limit of quantitation (LOQ) was 5.00 mg a.i./L.
[2] Results were generated using MacQuan version 1.6 software in full precision mode. Manual calculations may differ slightly.

METHOD OUTLINE FOR THE ANALYSIS OF PFOS IN FILTERED SALTWATER

Prepare matrix fortification samples in filtered saltwater matrix by spiking the requisite volume of PFOS stock solutions directly into filtered saltwater. Perform fortifications with gas-tight syringes and class A volumetric flasks. The matrix blank is unfortified filtered saltwater.

↓

Prepare appropriate dilutions of study and QC samples to within range of the PFOS HPLC/MS methodology: Partially fill Class A volumetric flasks with 50% methanol : 50% NANOpure® water dilution solvent containing 10.0 µg 4H PFOS/L and 0.05% (v/v) formic acid. Add appropriate volume of sample and bring the flask to volume with dilution solvent. Process the matrix blank samples using the same dilution and aliquot volume as for the lowest fortification level. Mix well by several repeat inversions.

↓

Ampulate samples and submit for HPLC/MS analysis.

Figure 1. Analytical method flowchart for the analysis of PFOS in filtered saltwater.

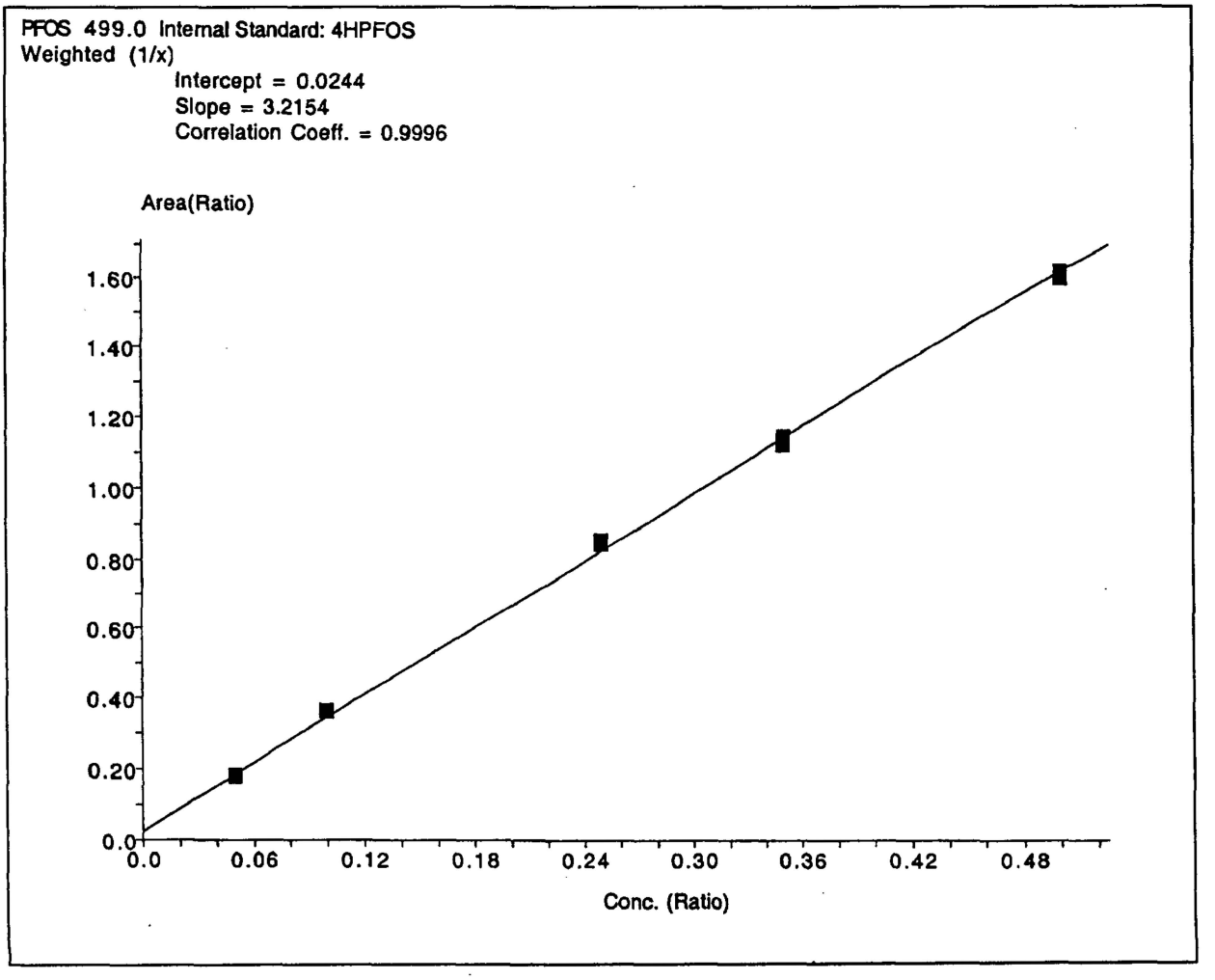

Figure 2. A typical calibration curve for PFOS.

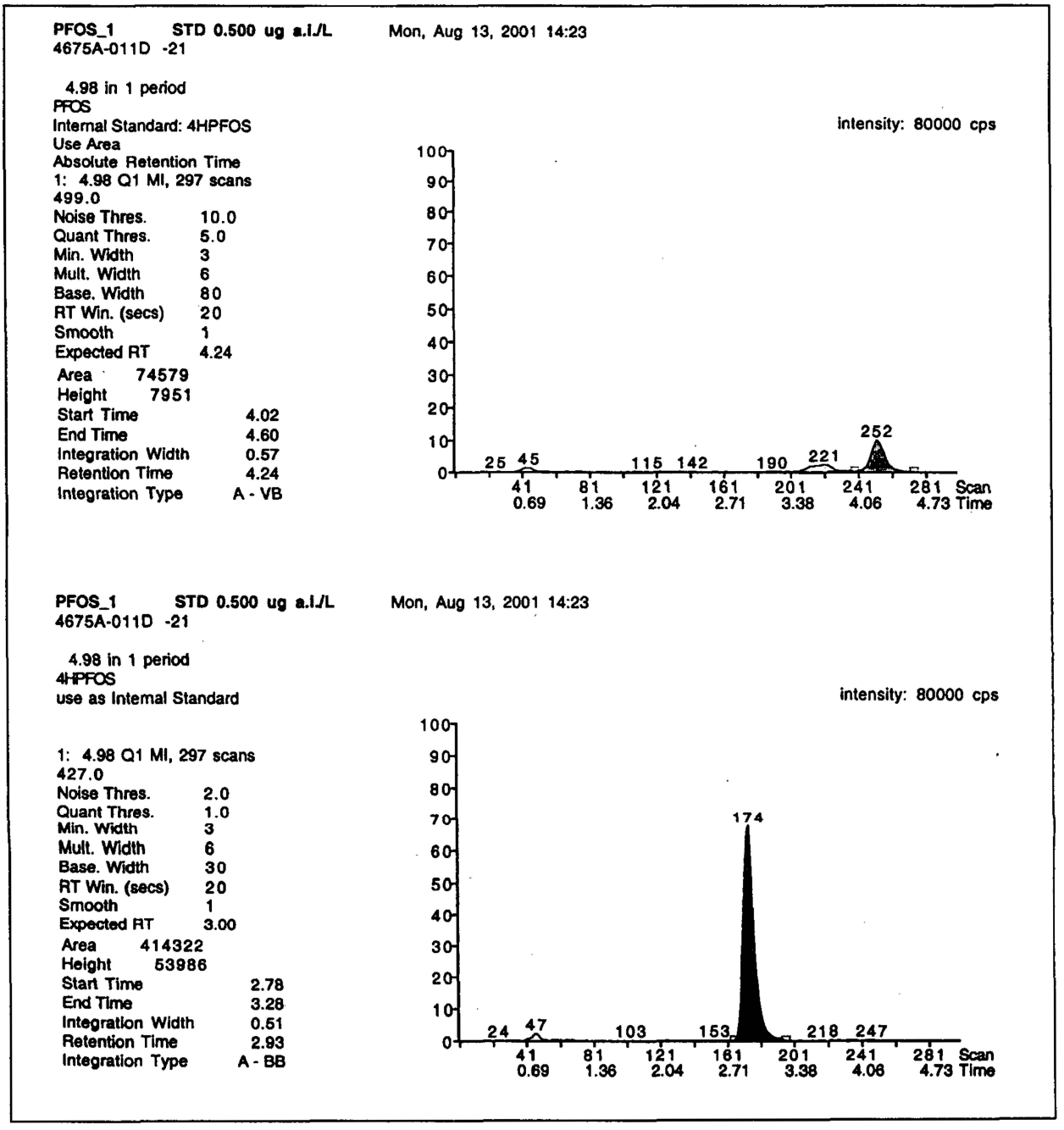

Figure 3. A representative ion chromatogram of a low-level (0.500 µg a.i./L) PFOS standard. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

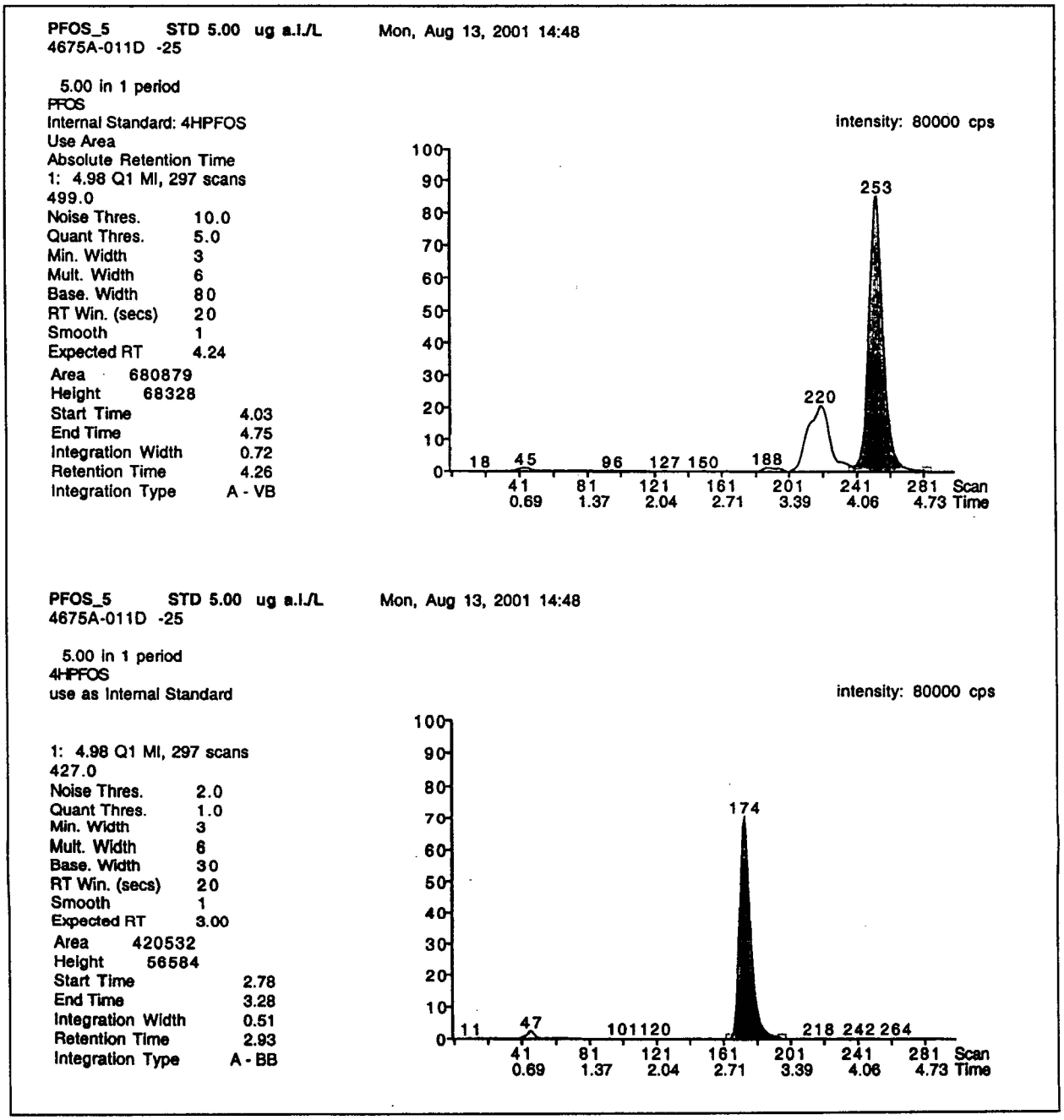

Figure 4. A representative ion chromatogram of a high-level (5.00 µg a.i./L) PFOS standard. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

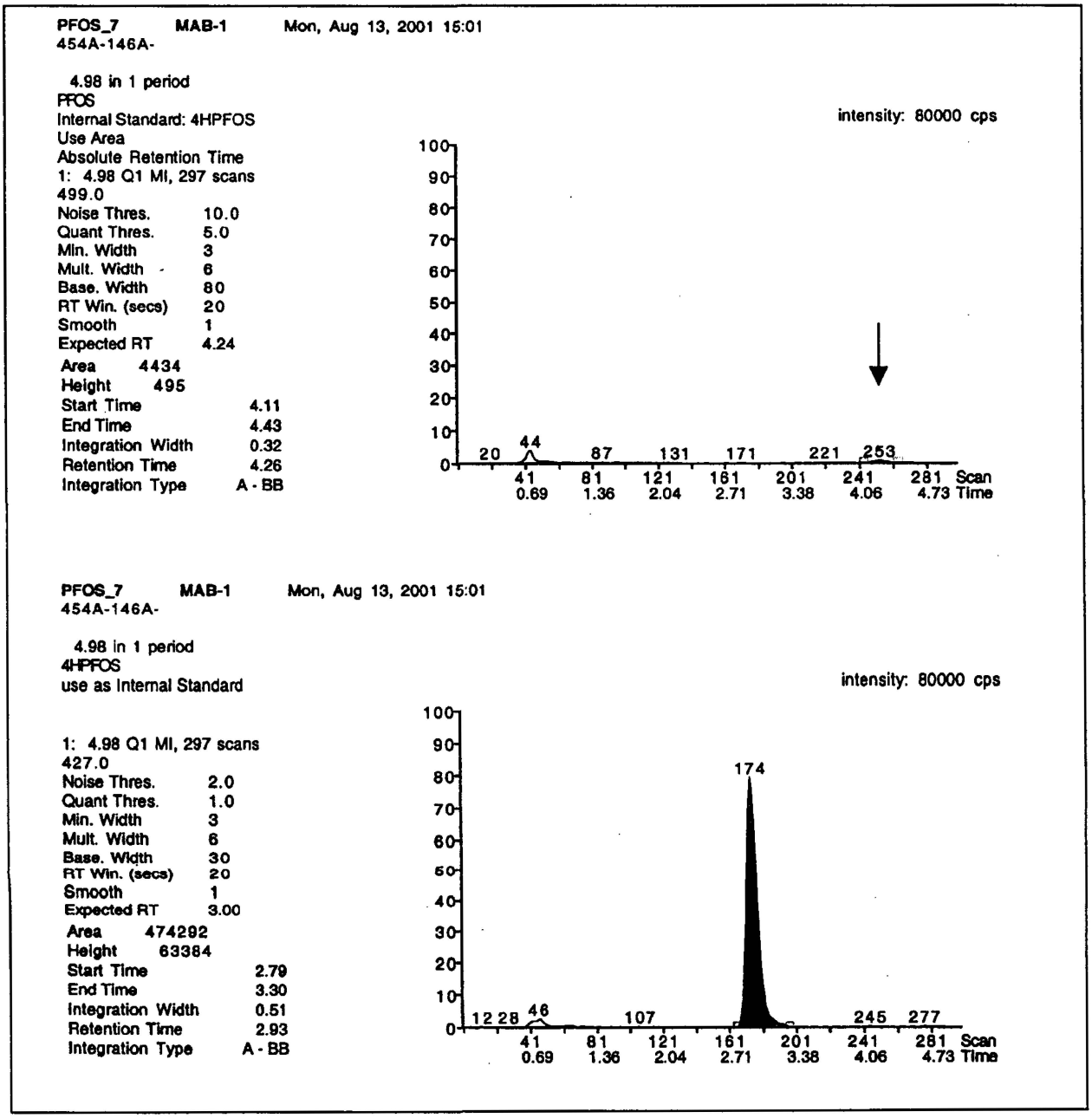

Figure 5. A representative ion chromatogram of a matrix blank sample (454A-146A-MAB-1). Dilution factor = 10,000x. The arrow indicates the retention time of PFOS. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

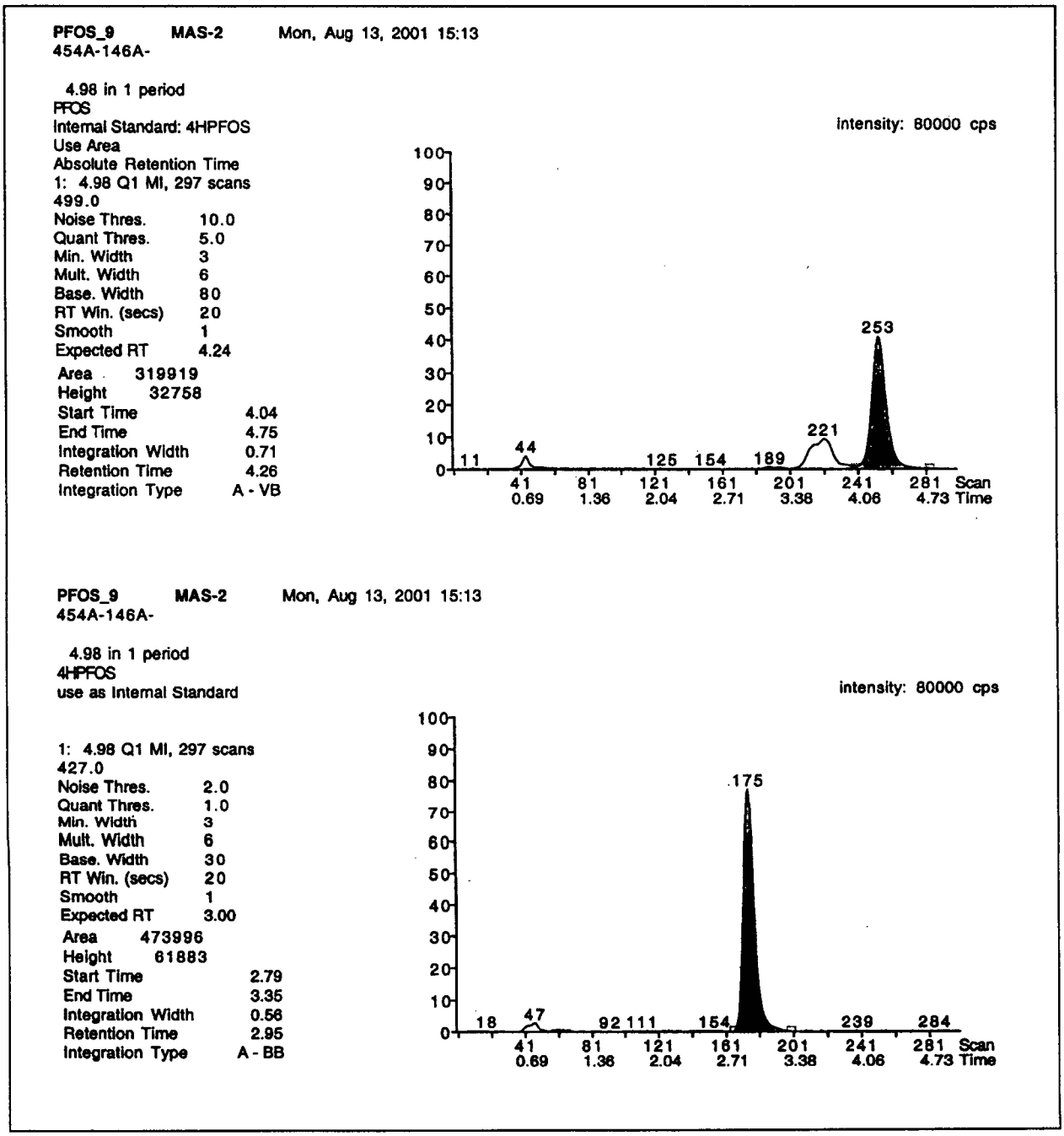

Figure 6. A representative ion chromatogram of a matrix fortification sample (454A-146A-MAS-2). Nominal Concentration = 22 mg a.i./L, Dilution factor = 10,000x. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

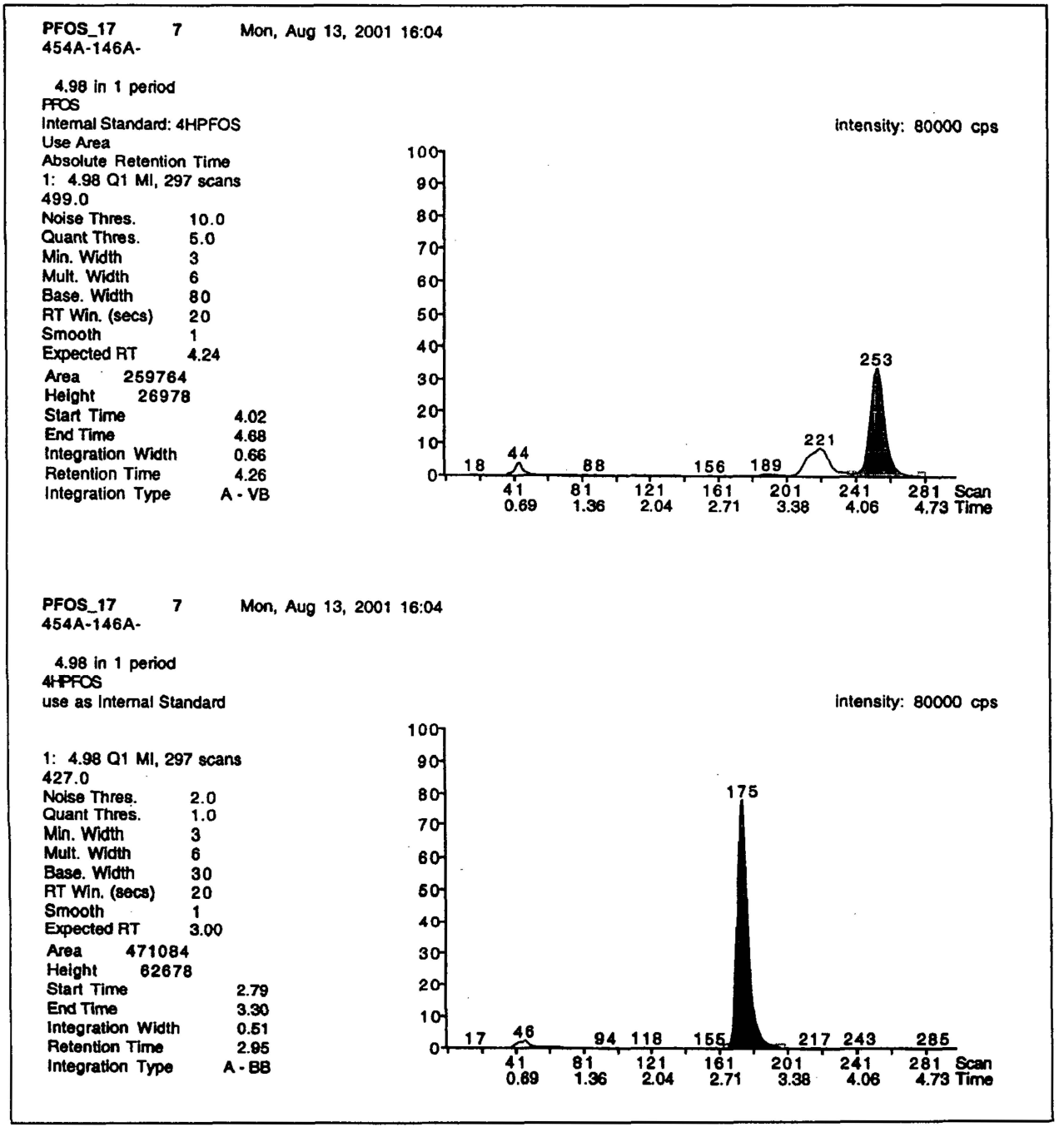

Figure 7. A representative ion chromatogram of a test sample (454A-146A-7). Nominal Concentration = 20 mg a.i./L, Dilution factor = 10,000x. (monitored masses = 499 amu (PFOS – top) and 427 amu (4HPFOS internal standard – bottom)).

Appendix 4

Changes to Protocol

This study was conducted in accordance with the approved Protocol with the following changes:

1. The protocol was amended to add the proposed experimental start and termination dates, test concentrations, and test and reference substance identification.

2. The protocol was amended to include the procedures and sampling scheme for conducting a limit test.

3. The protocol was amended to delete the analysis of feed since historical analyses of Wildlife International, Ltd. aquatic feed have shown that no contaminants are present at levels known to be capable of interfering with the study.

4. The protocol was amended to clarify the analytical methodology to be used during sample analyses.

5. The protocol was amended to change the proposed test dates and to include the procedures and sampling scheme to be used during a static-renewal test since the test was repeated under static-renewal test conditions to maintain test concentrations during the exposure period.

6. The protocol was amended to include the use of methanol in the preparation of the test solutions, and to include a solvent control group.

7. Salinity of the dilution water was measured at test termination, as well as at test initiation. Since the additional measurement confirmed that the salinity remained constant during the test, this change to the protocol had no impact upon the study

Appendix 5

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants
2. Willard B. Nixon, Ph.D., Director, Analytical Chemistry
3. Cary A. Sutherland, Laboratory Supervisor
4. Raymond L. Van Hoven, Ph.D., Scientist
5. Susan J. Palmer, Senior Biologist
6. Michelle Stence, Biologist
7. Frank J. Lezotte, Chemist